Spring 1992

T-cell Tumor Prevention by Treatments with Psoralen/UVA-Inactivated Tumor Cells

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A Thesis Submitted to the Yale University School of Medicine
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Medicine

by
Francis M. Lobo
1992

ACKNOWLEDGMENTS

I wish to thank Dr. Maritza Perez, my laboratory supervisor, for her friendship and superb guidance throughout, and Dr. Richard Edelson, chairman of the Department of Dermatology, for the rich opportunities that he has given me and for the interest that he has shown in my development as a physician and as a scientist.

I am also grateful to the many people who have made my work such a joyful undertaking: to Ms. Lori John, with whom I share countless happy memories; to Ms. Lissette Perez, whose friendship and help in the laboratory have been invaluable; to Dr. Yasuhiro Yamane, who performed much of the preliminary work in this model; to Ms. Venus Pitts, who always reminded me to have fun; and to Ms. Kathy Keane, without whose help this thesis probably would have been written in crayon. I must also thank the faculty and staff of the Department of Dermatology, who always will be the subject of warm reminiscences.

Finally, I must acknowledge the contributions of my family, not because I think that I actually can express in words such a profound degree of gratitude, but because it seems customary to do so.

TABLE OF CONTENTS

T CELL TUMOR PREVENTION BY TREATMENTS WITH PSORALEN/UVA-INACTIVATED TUMOR CELLS. Francis M. Lobo, Yasuhiro Yamane, Richard L. Edelson, and Maritza I. Perez. Department of Dermatology, Yale University, School of Medicine, New Haven, CT.

ABSTRACT

In the photopheresis therapy for cutaneous T-cell lymphoma, malignant T-cells exposed to 8-methoxypsoralen (8-MOP) activated by ultraviolet A (UVA) light appear to induce an immunologic response against the malignancy. In order to investigate this response, we used T-cell hybridoma 2B4.11 in genetically compatible (AKR X B10.A)F1 hybrid mice as a murine model for T-cell lymphoma. We compared the ability of cells inactivated by 8-MOP/UVA, mitomycin C (MMC), glutaraldehyde, and X-irradiation to immunize against tumor.

[^{3}H]-thymidine-uptake assays determined the doses of 8-MOP/UVA, MMC, glutaraldehyde, and X-irradiation necessary for inactivation of 2B4.11 cells. Mice then were subjected to an immunization protocol consisting of four weekly intraperitoneal injections of $5x10^6$ inactivated 2B4.11 cells followed by challenge with $5x10^6$ viable 2B4.11 cells. Of mice challenged 6 days after the end of the immunization protocol, only the recipients of cells inactivated by 8-MOP/UVA and MMC showed a significant enhancement of 80-day survival (50% alive in 8MOP/UVA group, 40% in MMC group, 0% in control group; $p<0.05$). Mice treated with 8-MOP/UVA-inactivated cells were challenged 30 days after the end of the protocol; enhancement of 80-day survival was very significant (87.5% alive vs. 0% in controls; $p<0.01$). Mice identically treated were challenged either with 2B4.11 or with related hybridoma C10.9; there was no significant cross-protection. C3H nu/nu athymic mice were subjected to the same immunization protocol; only recipients of cells damaged by X-irradiation demonstrated significant enhancement of 80-day survival (75% vs. 0% in controls). No cytotoxic cells were evident among splenocytes taken directly from immune mice or after *in vitro* stimulation with irradiated 2B4.11 in ^{51}Cr-release assays.

We conclude that 8-MOP/UVA-inactivated 2B4.11 cells induce an immune response that is more active at thirty days after immunization than at six days; is not significantly cross-protective against hybridoma C10.9; and appears to be at least partially mediated by non-T-cell elements.

Introduction

Photopheresis, the extracorporeal exposure of a patient's peripheral blood lymphocytes to ultraviolet A (UVA) light in the presence of 8-methoxypsoralen (8-MOP) with subsequent reinfusion into the patient, has become standard therapy for patients suffering from leukemic cutaneous T-cell lymphoma (CTCL) since its introduction by Edelson et al. in 1987 (1). A majority of CTCL patients who undergo photopheresis demonstrate a profound clinical response ranging from significant regression of cutaneous tumor burden to complete remission (1,2). Moreover, because the cytotoxic effects of 8-MOP are limited to those tissues that are exposed simultaneously to UVA, photopheresis lacks the severe side effects such as bone marrow depression and hair loss that are common to standard systemically-acting chemotherapeutic agents.

Despite the proven clinical efficacy and widespread use of photopheresis for CTCL, the mechanism of its anti-tumor effect remains obscure. Because the photopheresis procedure exposes only thirty to fifty percent of a patient's peripheral blood lymphocytes to UVA-activated 8-MOP, and responding patients subsequently experience a persistent elimination of tumor cells that could not have been inactivated directly by 8-MOP and UVA, it was hypothesized that photopheresis might induce a vaccination phenomenon in which the fraction of malignant T-cells inactivated by photopheresis provoked an immunological reaction against the remaining, unaffected malignant clones (1). This hypothesis found

support in work from other investigators in which vaccination of rodents with disease-mediating T-cell clones that had been lethally damaged could protect these animals against subsequent induction of disease by these same T-cell clones (3,4).

In order to study the mechanism by which photopheresis regulates T-cell proliferation in CTCL, a number of models of primarily T-cell-mediated immune phenomena have been subjected to 8-MOP/UVA manipulation. Perez et al. have developed a murine model in which lymphocytes mediating skin allograft rejection are exposed in vitro to 8-MOP/UVA and infused into syngeneic mice. The recipients demonstrate specific suppression of the activity of these lymphocytes as manifested by prolonged survival of the skin allograft against which the 8-MOP/UVA-inactivated lymphocytes were directed. They also demonstrated hyporesponsiveness in delayed type hypersensitivity assays, in mixed leukocyte culture, and in cytotoxicity assays against alloantigens associated with the allograft (5,6,7). Berger et al. have found that prophylactic infusions of 8-MOP/UVA-treated autoimmune effector lymphocytes in murine lupus can suppress significantly T-cell related features of the disease (8,9,10). In addition, photopheresis has been shown to enhance survival of cardiac xenografts in a primate model when used in conjunction with standard, non-specific immunosuppressants (9,11,12). These cumulative studies have given strong support for the theory targeting of expanded clones of T-cells as the primary mechanism of action of photopheresis. However, the ability to investigate this phenomenon at the level of cell-cell interactions has been hampered by the restriction of most of this research to

poorly characterized, oligoclonal or polyclonal populations of proliferating T-lymphocytes.

The successful fusion of antigen-specific T-cells with T-cell tumor lines to produce immortal T-cell hybridomas has provided an extremely useful tool for the study of clonally proliferating T-cells (13-16). The finding by Ashwell *et al.* that *in vitro* stimulation of certain of these hybridomas with their respective antigens results in a direct inhibition of their growth (17) led him to test for the ability of antigen stimulation to inhibit the growth of these hybridomas *in vivo* (18). When introduced into genetically compatible mice, these hybridomas caused a rapidly progressive lymphoma with internal organ metastases leading to death of the animals within six weeks. However, if these animals were given the hybridoma along with its appropriate antigenic stimulus, they were protected against subsequent tumor formation.

Using this murine model of a T-cell lymphoma, we attempted to determine whether prophylactic treatments with 8-MOP/UVA-inactivated hybridoma cells can protect mice against tumor development upon subsequent challenge with viable hybridoma cells. We compared cellular inactivation by 8-MOP/UVA with inactivation by X-irradiation, by mitomycin-C incubation, and by glutaraldehyde fixation in terms of inducing both short-term and long-term protection against tumor formation. This required the initial establishment of titration curves to ascertain the minimum dose necessary for cellular inactivation by these four modalities. We also treated a cohort of nude (athymic) mice in a similar manner in order to determine whether T-cell-dependent immunity plays a

role in protection against tumor development.

Photopheresis. Extracorporeal photochemotherapy, or photopheresis, exploits the unique ability of psoralen compounds to form powerful biologically active agents upon exposure to light radiation. Photopheresis treatment of cutaneous T-cell lymphoma requires the withdrawal of a patient's psoralen-containing blood, the extracorporeal separation of the peripheral blood lymphocyte fraction, exposure of this fraction to ultraviolet A light, and subsequent reinfusion into the patient. While exposed to UVA light, the psoralen molecules are transformed from a biologically inert state into highly active molecules that exert their effects throughout the cell by interacting with cellular DNA, proteins, and lipids (19).

Biochemical characteristics of psoralens. Psoralens are members of a class of compounds known as furocoumarins. They occur widely in nature, and are present in a variety of fruits, vegetables, and plant foliage, including such common sources as limes and the leaves and fruit of the fig tree. Furocoumarins are planar tricyclic aromatic compounds formed by the fusion of a furan ring to a coumarin molecule (19). The linear molecules formed by the fusion of the 2,3 furan bond to the 6,7 coumarin bond comprise the psoralen subgroup of furocoumarins. It was recognized for some time that 8-MOP, when activated by ultraviolet light in the 320 to 400 nm range, could form covalent monoadducts with thymine bases of DNA and, when intercalated between DNA strands, could form cross-linking bifunctional adducts between opposing pyrimidine bases (20-23). The excitation of the psoralen molecule by UVA

light allows monoadduct formation either at the 3,4 bond of the pyrone ring or at the 4',5' bond of the furan ring, while bifunctional crosslinking results from adduct formation at both sites of the psoralen molecule (19). Adduct formation is possible only when psoralen is in the excited state, and when exposure to UVA light is discontinued, nonadducted psoralen molecules revert instantly to their inert state (24).

This well known property of ultraviolet light-activated psoralens made it seem likely that their primary site of action was in the nucleus, the main repository of cellular DNA. However, the extensive distribution of the lipophilic psoralen molecules throughout the cell raised the possibility they were acting at other sites (25). Cell membrane-associated DNA (cmDNA), described by Bennett et al. (26), has been shown by Gasparro et al. to be a target of 8-MOP photoadduct formation (27). In addition, UVA-activated 8-MOP has been demonstrated to cause lipid oxidation through production of superoxide radicals (28), as well as peroxidation of low density lipoproteins in plasma (29). These photoperoxidized lipoproteins have been found to be selectively cytotoxic in vitro to a line of neoplastic CD4+ T cells derived from a patient with CTCL. Photoactivated 8-MOP also has been observed to bind nonspecifically to cellular proteins, although without apparent crosslink formation (19). Any or all of these cell-altering effects of 8-MOP/UVA may contribute to the clinical phenomena induced by photopheresis.

Early therapeutic uses of psoralens. The therapeutic virtues of these substances have been appreciated since antiquity; the

Ayurvedic system of medicine of ancient India used a plant now identified as Psoralea corylifolia (hence the word "psoralen") to treat areas of pigment loss. Similarly, the ancient Egyptians ingested the leaves of the plant Ammi majus while exposing depigmented areas of skin to sunlight in order to restore normal color (30). Both of these plants are rich sources of psoralens, and the latter became the object of intense studies in the 1940s by Fahmy, Abu-Shady, and El Mofty to determine its usefulness in the treatment of "leukoderma" or vitiligo (31,32). This work resulted in the identification of a previously isolated compound, 8-methoxypsoralen, as the active ingredient of Ammi majus as well as the finding that oral ingestion of this purified compound together with ultraviolet light exposure led to repigmentation in over 75 percent of cases of leukoderma. Lerner and Fitzpatrick refined these studies in the 1950s, establishing guidelines for the safe and effective use of 8-MOP and ultraviolet light in the treatment of vitiligo (33).

In the early 1970s, the use of 8-MOP and ultraviolet light for the treatment of psoriasis was explored in Europe and in the United States (34,35,36). Parrish et al. reported complete clearing with minimal side effects in all of twenty-one patients with generalized psoriasis with minimal side effects using oral 8-MOP and ultraviolet light (37). In this report, Parrish et al. demonstrated a superior clinical effect from the activation of 8-MOP by longwave ultraviolet (ultraviolet A) light than by conventional ultraviolet light. The effect of this therapy, for which Parrish et al. invented the term "photochemotherapy," was

attributed to the then recently discovered ability of ultraviolet light-activated 8-MOP to inhibit DNA synthesis. Psoralen and UVA, or "PUVA," therapy remains among the standard treatments for generalized psoriasis.

Psoralens and CTCL. Cutaneous T cell lymphoma is a malignant, clonal neoplasm of thymus-derived, CD4+ lymphocytes that preferentially infiltrate the skin before progressing to involve lymph nodes, internal organs, and peripheral blood. In distinction from most B cell lymphomas, bone marrow involvement occurs only late in the course of the disease. Mycosis fungoides and the Sezary syndrome represent overlapping phases of this single disease entity (38); together they have an incidence of approximately 800 to 1,000 new cases per year in the United States, making it a relatively uncommon malignancy (39). However, a number of other T cell lymphomas with skin involvement, including erythrodermic subacute and chronic T cell leukemia, lymphoma cutis, and lymphomatoid papulosis, have been identified as variants of the same disorder (40). By this more encompassing definition, the incidence of CTCL surpasses that of Hodgkin's disease, making it the most common adult lymphoma (41).

The first application of psoralen photochemotherapy to CTCL was an extension of the PUVA therapy for psoriasis, involving oral administration of 8MOP with subsequent external body UVA irradiation. Gilchrest et al. reported in 1976 that all of nine mycosis fungoides patients with disease resistant to other forms of treatment improved with PUVA therapy, with four patients experiencing complete clearing of skin manifestations (42). In

addition, response to PUVA was found to be of significant duration (43). The mechanism of this therapy was presumed to lie in the known DNA-binding action of UVA-activated 8-MOP as well as the relative sensitivity of T lymphocytes to ultraviolet light (44), which together resulted in direct mitotic inhibition and killing of malignant T cells in the skin.

Shortly before the trial of PUVA for CTCL, Edelson et al. described the successful management of a patient with the Sezary syndrome by leukapheresis (45). This therapy reduced the total body load of malignant T cells by nonspecifically removing peripheral blood mononuclear cells from the leukemic patient. The temporary clearing of skin involvement in this patient was attributed to a migration of T cells from the extravascular compartment into the intravascular compartment, restoring an equilibrium that had been disrupted by the depletion of the peripheral blood lymphocyte population. Although free of the toxicities associated with standard chemotherapies, this therapy, because of the inexact and nonspecific separation of leukocytes from peripheral blood, led to complications of anemia and leukopenia after multiple leukaphereses (24).

The combination by Edelson et al. of these two treatments for CTCL resulted in the development of the therapy now known as photopheresis. Combining the exquisitely controllable toxicity of UVA-activated 8-MOP with the ability of leukapheresis to separate leukocytes from peripheral blood allowed the selective photochemotherapeutic targeting of the leukemic cells of CTCL. The apparatus developed by Edelson and his colleagues allowed

withdrawal of 8-MOP-containing blood from the CTCL patient, separation of the leukocyte fraction, exposure of this fraction to UVA light, and subsequent reinfusion of all components into the patient. With this combination of techniques, the patient was to be spared the anemia that complicated multiple leukaphereses while benefiting from the effective elimination of malignant leukemic T cells.

The efficacy of UVA-activated 8-MOP for the *in vitro* inactivation of human lymphocytes had been demonstrated previously by Kraemer *et al.* (46,47). During the phase I trials for photopheresis, Berger *et al.* extended these investigations to determine a dose response curve for the inhibition of lymphocyte proliferation (48). Her work determined that the average serum level of 8-MOP achieved in the patient undergoing photopheresis, 100 ng per milliliter, combined with 1 J per cm^2 of UVA was more than sufficient to damage lymphocytes lethally as shown by the abrogation of lymphocyte response to phytohemagglutinin.

In 1987, Edelson *et al.* reported the results of a multicenter trial of photopheresis for the treatment of patients with the erythrodermic CTCL, a form of the disease with a relatively poor prognosis and the same form that Edelson had treated earlier with leukapheresis (1,45). Of the 37 patients who completed the trial, 27 responded to photopheresis with an average decrease in skin involvement of 64 percent. These responders included 20 of 28 patients who had been resistant to standard chemotherapy for CTCL. Side effects associated with the therapy were minimal; the hair loss, bone marrow suppression, and gastrointestinal erosions common

to standard chemotherapy did not occur. Similar results were reported by Heald et al. in a follow-up study in 1989 (2).

Most interesting, however, were the long term remissions off photopheresis therapy of a group of responders, including two with quiesence of disease lasting for more than two years at the time of Edelson's report. While the initial response to therapy could have been due to direct toxicity of UVA-activated 8-MOP to the malignant clones, this long-term response suggested another mechanism. It was hypothesized that this effect might have been caused by an immunologic response against the 8-MOP-inactivated lymphocytes, of which the single most populous clone was likely to be that of the malignancy. Theoretical support for this hypothesis came from observations by Edelson (24) and Heald (2) that CTCL patients with relatively intact immune systems, usually those with the shortest time interval between diagnosis and onset of treatment and with near normal CD4/CD8 ratios of blood lymphocytes, had the best and most enduring responses to photopheresis.

The success of photopheresis in controlling the malignant proliferation of T cells in CTCL inspired clinical trials to explore its efficacy in controlling aberrant proliferations of T cells that might underlie such autoimmune disorders as scleroderma (49,50), pemphigus vulgaris (51,52), and rheumatoid arthritis (53). The early results of these trials have shown that photopheresis has a beneficial effect on all of these autoimmune disorders. In addition, photopheresis recently has been used experimentally with appaerent success to control rejection of human cardiac allografts (54).

Regulation of T cell proliferation by photopheresis. The hypothesis of immunization against proliferating T cell clones as a mechanism of the action of photopheresis also found support in the findings of Cohen et al. from their work with lymphocytes mediating autoimmune disease (3,4). Cohen's group used as its model for autoimmune disease experimental autoimmune encephalomyelitis (EAE), an acute paralytic syndrome that can be induced in laboratory animals by immunizing them with myelin basic protein (MBP) emulsified in complete Freund's adjuvant. Having isolated in vitro T cell lines reactive with MBP from rats affected with EAE, Cohen et al. were able to induce clinical EAE in naive rats by intravenous infusion of these same MBP-reactive T cell lines, without requiring immunization with MBP. In this way it was demonstrated that EAE was mediated solely by MBP-reactive T cells.

Cohen's group next demonstrated that they not only could induce EAE by infusing living MBP-reactive T cells, but, by inoculating animals with the same T cell lines inactivated by gamma irradiation or by mitomycin C, they also could protect animals against subsequent induction of EAE. They termed this phenomenon "T cell vaccination," for they had induced immunity against the disease caused by MBP-reactive T cell lines by inoculating animals with the inactivated forms of these same cells, in a manner analogous to the prevention of infectious diseases by immunization with attenuated forms of the etiologic agents. They further showed that this immunity was specific for the idiotype of the clone used for vaccination. That is, a T cell line reactive with one epitope of MBP induced an immunity that did not protect against disease

induction by another T cell line that reacted with a different epitope on the MBP molecule. The idiotype specificity strongly suggested that the T cell receptor itself was the target of the immune response in vaccinated animals.

Cellular inactivation by UVA-activated 8-MOP was tested subsequently in the EAE system by Khavari from Edelson's group (55). His work demonstrated that cells inactivated by 8-MOP/UVA were more effective in vaccinating against EAE than those inactivated by hydrostatic pressure or by glutaraldehyde, the two most effective methods of attenuation to be found by Cohen's group (56,57). This evidence suggested that photopheresis might function to provide a safe and practical method for extracorporeal attenuation of the malignant clones of CTCL that, upon reinfusion into the patient, induced a vaccination effect against all clones of the malignancy (58). As in the EAE system, a good candidate for the target of this immune reaction was the T cell receptor, a unique antigen carried by all of the malignant clones.

Work in other animal systems also pointed to an immunologic reaction against activated T cells as a central mechanism in photopheresis. Laroche et al. raised an expanded population of effector T cells by injecting mice with sheep red blood cells (59). After inactivating in vitro splenocytes taken from these injected mice with 8-MOP/UVA, they infused these cells into syngeneic recipients. These recipients demonstrated an inhibition of response specifically to sheep red blood cells upon subsequent delayed type hypersensitivity challenge, compared with untreated controls. It was demonstrated further that this treatment was

ineffective if the recipients were given inactivated splenocytes from naive mice which did not contain an expanded population of specifically reactive lymphocytes. This indicated that although a mixed population of lymphocytes was used, the suppressive effect was directed specifically against the expanded clone or clones in an idiotypic manner.

Perez et al. found that they were able to use photopheresis to suppress the T cell-mediated rejection of allografts in a murine model (5-7). In this system, splenocytes from mice acutely rejecting skin allografts, containing expanded populations of effector T cells that mediated the graft rejection, were inactivated in vitro by UVA-activated 8-MOP and then infused into naive syngeneic mice. Upon subsequent allografting, recipients of these inactivated effector lymphocytes demonstrated prolonged survival only of the allograft that had been the target of the inactivated lymphocytes, but not of an irrelevant, previously unseen allograft. This specific suppression of response against alloantigen in vivo correlated with inhibition in vitro of the mixed leukocyte culture response, the cytolytic T cell response, and the delayed type hypersensitivity response. Adoptive transfer experiments later revealed that the suppression of alloreactivity could be transferred to naive syngeneic mice by Thy-1+, Lyt-2+, L3T4- lymphocytes (60). It appeared that recipients of 8-MOP/UVA-inactivated lymphocytes were vaccinated specifically against the activity of the alloreactive effector T cells, and that this immunity was mediated by phenotypically suppressor/cytotoxic T lymphocytes.

Other animal models gave further support for the supression of proliferating T cells by photopheresis. Berger et al. employed the MRL/l mouse, which with increasing age develops a syndrome similar to systemic lupus erythematosus, consisting of the primary proliferation of phenotypically aberrant inducer T cells with subsequent lymphoid hyperplasia, B-cell hyperactivity, and autoantibodies leading to immune complex formation and ultimately fatal immune complex glomerulonephritis (8-10). By prophylactically treating young MRL/l mice with 8-MOP/UVA-inactivated splenocytes taken from older, diseased syngeneic animals, Berger was able to prolong survival and to inhibit significantly several parameters of the disease process, including the abnormal proliferation of Thy-1+ lymphocytes, the progression of lymphoid organomegaly, the development of autoantibodies, and the loss of response of T cells to mitogen that was seen normally in untreated mice.

The animal models used by Laroche, Perez, and Berger, as well as the clinical experience with CTCL patients, proved that it was not necessary to isolate the pathogenic or malignant T cell clone in vitro in order to "vaccinate" against it in vivo, as was true in the EAE system. This of course was the great virtue of photopheresis in terms of its clinical applicability. However, finer dissection of the immune response elicited by photopheresis would require the use of well characterized, clonal populations of T cells. Investigations of theoretical antiidiotypic responses in particular would necessitate the use of clones with known antigenic determinants. One avenue for these investigations could be the

further use of T cell lines such as the EAE-inducing clones developed by Cohen et al., long term cultures of normal, antigen-specific T cells maintained in vitro by repeated stimulation with antigen and interleukin-2 (IL-2). Another tool for these studies, however, could be the immortal, spontaneously and clonally proliferating, antigen-specific products of the fusion of normal T cells and T cell tumor lines, known as T cell hybridomas. The latter approach is the subject of the experiments described in this thesis.

T cell hybridoma 2B411 and a murine model for T cell lymphoma. In 1975, Kohler and Milstein described a technique for the fusion in vitro of antigen-specific B cells with a spontaneously proliferating myeloma cell line to produce B cell hybridomas, immortal cell lines that produce immunoglobulins determined by the antigen specificity of the B cell parent (13). Shortly thereafter, successful fusions of T cells with T cell tumor lines produced analogous hybridomas (14,15), and in 1981, Kappler et al. reported the first such T cell hybridomas with known antigen specificity (16). These hybridomas were MHC class II restricted, and secreted IL-2 upon stimulation with antigen and class II.

Ashwell et al. subsequently reported on the surprising effects of antigen stimulation on T cell hybridomas using a panel of hybridomas of known antigen specificity (17). He found that in addition to production of IL-2, activation of T cell hybridomas with antigen resulted in a two phase inhibition of the normal spontaneous proliferation of the hybridoma: an initial block in the cell growth cycle at the G_1/S interface detected within one hour

of activation, and a second phase of calcium-dependent cell lysis at four to six hours from activation (61). Inhibition of T cell hybridoma growth associated with IL-2 production was also achieved by monoclonal antibodies directed against the activating molecules CD3, Thy-1, and Ly-6 (62), although activation via the latter two molecules required the coexpression of CD3 and the T cell receptor molecules (63).

The intruiging finding of inhibition of T cell hybridomas via antigen activation led Ashwell and his colleagues to test for the ability to arrest the growth of these hybridomas *in vivo* (18). Ashwell *et al.* used two hybridomas created by the fusion of antigen-primed T cells from B10.A mice with cells of the AKR mouse thymoma line BW5147. The first, 2B4.11, had antigen specificity for pigeon cytochrome c, and the second C10.9, was specific for antigen hen egg lysozyme (HEL). When injected subcutaneously into genetically compatible (AKR x B10.A)F1 hybrid mice, these hybridomas caused progressive tumors that led to the death of the host animals within forty days. However, if the appropriate antigen was administered intraperitoneally to the animals at the time of hybridoma inoculation, the mice were protected against subsequent tumor development. Administration of antigen after the tumors had become established almost invariably led to the resolution of the primary tumor mass and to long term survival in a substantial fraction of mice.

Of great interest was the additional finding that long term survivors of antigen activation therapy were protected against subsequent tumor formation upon rechallenge with the same

hybridoma. T cell-dependent immunity was implicated in this phenomenon by the finding that in genetically compatible nude (athymic) mice, antigen activation both was less efficient in the prevention of tumor and, in the few survivors of the initial inoculation, led to no long-term protection against rechallenge. However, this immunity was not specific for the hybridoma used in the initial inoculation, as the survivors of C10.9 inoculation with HEL treatments were cross-protected against tumor development when challenged with 2B4.11.

Bridges and Longo conducted further experiments to define the immune response involved in the cure of 2B4.11 tumor by antigen activation (64). Their elegant studies using in vivo depletion of T cell subsets revealed that the immunity induced in cured mice was mediated by CD8-bearing cells and was affected only minimally by depletion of CD4-bearing cells. Furthermore, in vitro studies with splenocytes from 2B4.11-immune mice revealed a CD4-/CD8+ population that was cytotoxic specifically to 2B4.11 cells but not to any other related hybridoma. However, in vivo rechallenge of 2B4.11-cured mice again demonstrated cross-protection against the related hybridoma C10.9, as well as against their parent thymoma BW5147, suggesting the induction of antitumor effector cells of broader specificity that conferred long-lasting protection in vivo but that were not propagated in vitro by the protocol used for these studies. Yet despite the unresolved mysteries, these experiments clearly described the development of specific immunity against a T cell tumor.

The objective of the experiments described in this thesis is to

help to establish the 2B4.11 hybridoma as a model of a T cell lymphoma that can be used to study the anti-tumor effects of photopheresis. Preliminary experiments in our laboratory have determined the number of hybridoma cells needed to induce a lymphoma that is rapidly fatal to genetically compatible mice. The first experiment described in this thesis defines the minimum inactivating dose for 2B4.11 of four modalities of cellular damage: UVA-activated 8-MOP, mitomycin C incubation, glutaraldehyde fixation, and X-irradiation. These forms of inactivation were chosen because they have been demonstrated by the experiments with the EAE system of Cohen et al. to be methods of T cell inactivation conducive to a vaccination phenomenon. Next a comparison is made of the efficacy of each of these methods of cellular inactivation in an immunization protocol consisting of four weekly intraperitoneal injections of inactivated 2B4.11 cells prior to challenge with viable hybridoma cells. Evidence of short-term protection against tumor development is sought by challenging mice six days after the final injection, while long-term protection is tested by challenge one month after the final injection. Finally, the importance of a functional T cell immunity for any observed protective effect is probed by subjecting nude (athymic) mice to the same protocol. The results of these experiments establish 8-MOP/UVA as an effective method of inducing protection against tumor in this well-characterized model of T cell tumor immunity.

MATERIALS AND METHODS

Mice.

(AKR x B10.A)F1 mice were bred at this institution from B10.A/SgSn (B10.A) and AKR mice obtained from the Jackson Laboratory, Bar Harbor, ME. C3H nu/nu mice were obtained from the Small Animal Section, Veterinary Resources Branch, National Cancer Institute, National Institutes of Health, Bethesda MD. All mice were housed in filter cages in a pathogen-free room.

Tumor cells.

T cell hybridomas 2B4.11, C10.9, and 2B4.11.21.2.2 were the generous gift of Dr. Jonathan Ashwell (National Institutes of Health, Bethesda, MD). Hybridomas were maintained in a growth medium consisting of RPMI 1640 with Hepes 25 mM (Mediatech, Washington, DC), supplemented with 10% heat-inactivated fetal calf serum (Biofluids Inc., Rockville, MD), 4 mM L-glutamine (Mediatech), 100 IU/ml pennicillin & 100 ug/ml streptomycin (Mediatech), 100 ug/ml gentamicin (Gibco, Grand Island, NY) and 50 uM 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA). AKR thymoma BW5147 was purchased from the American Type Culture Collection (Rockville, MD) and was maintained in a medium consisting of Dulbecco's modified Eagle's medium with 4.5g/l glucose (Gibco) and supplemented with 10% heat inactivated fetal calf serum (Biofluids) and antibiotics as listed previously. Research assistants Ms. Lori John and Ms. Lissette Perez assisted extensively in the maintainance of cell cultures.

Methods of hybridoma inactivation.

8-MOP/UVA. 8-MOP (Elder Pharmaceutical, Costa Mesa, CA) was dissolved initially at 10 ug/ml in 100% ethanol and later diluted in phosphate-buffered saline (PBS) to varying concentrations, for titration curves (see below), or to 200 ng/ml in PBS for the mouse treatment protocol. Concentrations of 8-MOP in PBS were verified by Mr. Louis A. Amici using reversed phase high pressure liquid chromatography as described elsewhere (65). 2B4.11 hybridoma cells were suspended at 1-1.5x10^6/ml in the appropriate dilution of 8-MOP and incubated for 20 min. at room temperature shielded from light. The cell suspension subsequently was dispensed into plastic petri dishes (Falcon, Becton Dickinson & Co., Oxnard, CA) and exposed to varying doses of UVA, for titration curves (see below), or to 2 J/cm^2 of UVA, for the mouse treatment protocol, in a specially constructed light box consisting of six black light flourescent tubes (FL40, Sylvania GTE Products, Danvers, MA) emmitting broad spectrum UVA energy (320-400 nm), filtered through a sheet of window glass to remove UVB-range energy. The UVA energy dose was calculated after measurement with a spectrophotometer (IL700A, International Light, Newburyport, MA). After irradiation, cells were washed twice with PBS to remove excess 8-MOP.

Glutaraldehyde. Glutaraldehyde (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 0.5% (w:v) for the cellular inactivation assay (see below) and for the mouse treatment protocol. 2B4.11 cells were suspended in this solution at 1-2x10^7 cells/ml and incubated at 37^{o}C under a humidified, 5%

CO_2 atmosphere for thirty minutes. The cells subsequently were washed three times in PBS to remove excess glutaraldehyde.

Mitomycin C. Mitomycin C (Sigma) was dissolved in PBS at 50 ug/ml for the cellular inactivation assay (see below) and for the mouse treatment protocol. 2B4.11 cells were suspended in this solution at $1\text{-}2x10^7$ cells/ml and placed in a 37^oC water bath for thirty minutes. The cells subsequently were washed three times to remove excess mitomycin C.

X-irradiation. Using a Gammacell 1000A ^{137}Cs-source cell irradiator (AECL Industrial, Kanata, Ontario, Canada), 2B4.11 cells were subjected to varying doses of X-irradiation, for titration curves (see below), or to 5,000 rads of X-irradiation for the mouse treatment protocol.

Titration curves for cellular inactivation

2B4.11 cells were subjected to a range of doses (see Tables 1 and 2) of the methods of cellular inactivation described above and resuspended at $2.5x10^5$ cells/ml in hybridoma growth medium. These cells were aliquotted at $5x10^4$ cells/well in triplicate 225 ul cultures in 96-well, round-bottom plates (Corning, Corning, NY). Cultures were maintained for up to ten days with daily changing of medium after the third day by gentle aspiration of supernatant and addition of 150 ul of fresh medium to each well. For the final 18 hours of incubation of each culture, wells were pulsed with 1 uCi of ^3H-thymidine (Amersham, Arlington Heights, IL); cultures then were harvested onto glass fiber filter strips with a semiautomated harvester (PHD Cell Harvester, Cambridge

Technology Inc., Cambridge, MA). Incorporation of ^{3}H-thymidine in each sample was determined in a liquid scintillation beta counter (RackBeta Spectral, LKB, Turku, Finland). Titration curves for the inactivation of 2B4.11 by glutaraldehyde and by X irradiation were performed by Dr. Maritza Perez and Dr. Yasuhiro Yamane.

Mouse treatment protocol

(AKR X B10.A)F1 hybrid or C3H nu/nu mice were given weekly intraperitoneal (i.p.) injections of 5 x 10^6 inactivated 2B4.11 cells suspended in 200 ul of PBS for four weeks. Either six days (testing for short-term protection) or thirty days (testing for long-term protection) following the fourth treatment, the mice were challenged with a subcutaneous (s.c.) injection of 5 x 10^6 viable 2B4.11 cells suspended in 100 ul PBS. Cells from the same syringe were used to challenge both experimental and control mice, and all mice received the challenge dose within 30 minutes of each other. Cells remaining in the syringe after challenge were assayed for viability by trypan blue exclusion and were found to be greater than 95% viable. The challenge dose of 5 x 10^6 2B4.11 cells was found to be the minimum necessary to kill 95% of naive (AKR X B10.A) mice in previous experiments conducted by Dr. Yasuhiro Yamane and Dr. Maritza Perez. Tumor area was determined on the indicated days after challenge by measuring two perpendicular diameters of the palpable tumor mass with a dial caliper (Monostat, Fisher Scientific, Springfield, NJ). Mice subsequently were monitored for survival. Approximately one-half of the mice in each

experimental and control group were treated, challenged, and monitored by Dr. Maritza Perez, Dr. Yasuhiro Yamane, and Ms. Lori John.

Assay for in vivo immune specificity

In order to determine whether the immunity against 2B4.11 was specifically protective against only this tumor cell, mice were pretreated with 8-MOP/UVA-inactivated 2B4.11 cells in a manner identical to the treatment protocol detailed above. Six days after the final treatment, mice were challenged either with 5×10^6 2B4.11 cells or with 10×10^6 C10.9 hybridoma cells. C10.9 is a T cell hybridoma with TCR specificity for hen egg lysozyme; like 2B4.11, it is the product of the fusion of an activated T lymphoblast from a B10.A mouse with the AKR thymoma BW5147. The challenge dose of 10×10^6 C10.9 cells was found to be the minimum necessary to kill 95% of naive (AKR X B10.A)F1 mice in previous experiments conducted by Dr. Yasuhiro Yamane and Dr. Maritza Perez. Tumor area was determined on the indicated days after challenge by the same methods as above, and mice were monitored for subsequent survival. All mice in this assay were treated, challenged, and monitored by Dr. Maritza Perez, Dr. Yasuhiro Yamane, and Ms. Lori John.

Cytotoxicity Assay

5×10^6 splenocytes from 2B4.11-immune mice were cultured with 5×10^5 irradiated (5000 rad) 2B4.11 cells for four days in 24 well plates. Viable effector cells then were separated via density centrifugation on Lympholyte-M (Cedar Lane Laboratories,

Hornby, Ontario, Canada) and were either tested directly for cytotoxic activity or were subjected to a second round of rest by culture of 1 x 10^6 effector cells with 4 x 10^6 irradiated (3300 rad) (AKR X B10.A)F1 splenocytes for five to ten days, followed by restimulation by culture of 4 x 10^5 rested effector cells with 4 x 10^5 irradiated (5000 rad) 2B4.11 cells and 4 x 10^6 irradiated (3300 rad) (AKR X B10.A)F1 splenocytes. These restimulated effector cells then were tested for cytotoxic activity. Alternatively, 2B4.11-immune mice, having survived tumor challenge by more than three months, were stimulated in vivo by two i.p. injections of 5 x 10^6 8-MOP/UVA-inactivated 2B4.11 cells eight and four days prior to harvesting of their splenocytes for propagation as above. A final 2B4.11-immune mouse was stimulated in vivo by subcutaneous injection of 5 x 10^6 8-MOP/UVA-inactivated 2B4.11 cells eight and four days prior to harvesting of its splenocytes for propagation as above. The medium used for propagation of effector cells consisted of 50% RPMI 1640 with Hepes 25 mM (Mediatech, Washington, D.C.) and 50% EHAA (Biofluids Inc., Rockville, MD.) supplemented with 10% heat-inactivated fetal calf serum (Biofluids Inc., Rockville, MD), 4 mM L-glutamine (Mediatech), 100 IU/ml penicillin & 100 ug/ml streptomycin (Mediatech), 100 ug/ml gentamicin (Gibco, Grand Island, N.Y.), and 50 uM 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA).

1 x 10^6 of each target cell (2B4.11, C10.9, BW5147, and 2B4.11.21.2.2, a T-cell receptor negative variant of 2B4.11) were suspended in 0.3 ml of fetal calf serum to which was added 100 uCi

of $Na_2{}^{51}CrO_4$ in a volume of 0.1 ml; the suspension was incubated at 37° C for 90 minutes with gentle vortexing every 10 to 15 minutes. Target cells were washed three times with cytotoxicity medium (RPMI 1640 supplemented with 0.3% bovine serum albumin and containing Hepes, L-glutamine, antibiotics, and 2-mercaptoethanol as above. Effector cells recovered from culture were resuspended in cytotoxicity medium and aliquoted in 96 well plates at the appropriate number for the desired effector:target ratio. Target cells were resuspended in cytotoxicity medium and added at 5×10^3 cells per well to the 96 well plates for a total volume of 0.2 ml per well. Plates were centrifuged at 500 X g for 1 minute prior to incubation at 37°C for 4 hours. After incubation, plates were centrifuged at 500 X g for 10 minutes, and 0.1 ml of supernatant from each well was harvested for counting in a gamma counter (1275 Minigamma, LKB, Turku, Finland).

Cytotoxicity was calculated by the formula:

$$\% \text{ cytotoxicity} = 100 \times \frac{(\text{experimental} - \text{spontaneous release})}{(\text{maximal} - \text{spontaneous release})}$$

Spontaneous release was determined by incubating target cells alone in cytotoxicity medium, while maximal release was obtained by incubating targets in medium containing 1% Triton X-100 detergent.

RESULTS

Inactivation of 2B4.11. Prior to starting inoculations of mice with inactivated 2B4.11 hybridoma cells, extensive titration curves were performed in order to determine the minimum necessary dose of 8-MOP/UVA, as well as the efficacy of previously established doses of the other cytotoxic agents, for the inhibition of spontaneous proliferation of hybridoma cells as measured by ^{3}H-thymidine uptake assays. Cultures were maintained and ^{3}H-thymidine uptake was measured for up to ten days after the time of cell damage in order to detect any recovery of non-lethally damaged cells or emergence of cell populations resistant to inactivation by 8-MOP/UVA.

Table 1 shows that inactivation of 2B4.11 can be acomplished by 8-MOP at a concentration of 100 ng/ml only when activated with 7 J of UVA light. Inactivation also is achieved by a combination of 8-MOP at 200 ng/ml with 2 J of UVA energy. Because the latter combination of drug and light more closely approximates the levels reached in clinical photopheresis (48), it was used to inactivate 2B4.11 in the current study. Table 1 also demonstrates the failure of non-activated 8-MOP at doses up to 1000 ng/ml as well as up to 3 J of UVA energy alone to inhibit cell proliferation. The lack of data on the inhibitory effect of 7 J of UVA energy alone makes it impossible to preclude the possibility that the inactivation seen with 100 ng/ml 8-MOP combined with 7 J of UVA energy was due in greater part to a cytotoxic effect of such a high degree of UVA energy.

Mitomycin C at 50 ug/ml, the dose used for the inactivation of T lymphoblast lines in the EAE model (3), was sufficient for inactivation of 2B4.11 (Table 2). Glutaraldehyde at 0.5% (w:v) was effective for inactivating 2B4.11, a greater concentration than was required in the EAE model (57). Finally, a dose of 5000 rads of X irradiation was found to be enough to inactivate 2B4.11.

Short term protection against tumor. (AKR X B10.A)F1 mice were subjected to a treatment protocol consisting of four weekly i.p. injections of 5×10^6 2B4.11 cells inactivated by the four modalities described above. Mice then were challenged on the sixth day following the last treatment by a s.c. injection of 5×10^6 viable 2B4.11 cells. As shown in Table 3, tumor growth in the first ten days after challenge was significantly impeded only in mice pretreated with cells inactivated by 8-MOP/UVA; treatments with cells inactivated by mitomycin C, by glutaraldehyde, and by X irradiation also impeded tumor growth to a lesser degree. The incidence of tumor formation was lower in all groups of pretreated mice, especially in the recipients of 8-MOP/UVA and mitomycin C-inactivated tumor cells, and tumors were observed to disappear over the first ten days in a small fraction of all experimental groups. Significant regression was noted in the 8-MOP/UVA and mitomycin C treatment groups. No such regression took place in the untreated control group. However, tumors that had not regressed in experimental mice by day ten were neither significantly smaller nor less lethal than those in control mice.

Pretreatment with cells inactivated by 8-MOP/UVA, by mitomycin C, and by X irradiation led to a significant improvement in 40-day

survival compared with untreated control mice; by 80 days, enhancement of survival was significant only for the 8-MOP/UVA (6/12 dead) and mitomycin C (6/10 dead) groups compared with the control group (10/10 dead). Mice pretreated with cells inactivated by glutaraldehyde also demonstrated enhanced 40-day and 80-day survival, but this protection was not statistically significant with the sample size in this experiment. Deaths both in the experimental groups and in the control group occurred predominantly within forty days of challenge.

The degree of enhancement of survival afforded by pretreatment with 8-MOP/UVA-inactivated cells appeared to surpass that of the other inactivating modalities, but once again this difference was not significant with the sample sizes of the experiment. These data show that pretreatment of mice with tumor cells inactivated by all four tested modalities confers some degree of immunity against the growth and mortality of the same tumor. This immunity is active at six days after the end of the treatment protocol. However, this immunity is effective only in a fraction of treated mice, and unprotected mice are overwhelmed by tumor at a rate similar to that seen in control mice. The data also suggest that 8-MOP/UVA is the most effective form of cellular inactivation for inducing this immunity.

Long term protection against tumor. In order to determine the longevity of the observed immunity against tumor, mice were treated with 8-MOP/UVA-inactivated tumor cells as above, but were not challenged until one month after the last treatment. As detailed in Table 4, these mice demonstrated both a significant decrease in

tumor incidence and a significant inhibition of tumor growth. The 80-day survival of treated mice (1/8 dead) was significantly higher than that of untreated controls (6/6 dead). Indeed, the enhancement of survival was significantly greater than that observed in the recipients of 8-MOP/UVA-inactivated cells with challenge at day six after the last treatment. Once again, the majority of deaths in the control group occurred within forty days of challenge, while no such rapid death was observed in the experimental group. These data indicate that the immunity against tumor induced by treatments with 8-MOP/UVA-inactivated tumor cells is more potent at thirty days following the treatment protocol than at six days, suggesting an interval evolution of the mechanism of the observed immunity against tumor.

Evidence of protection against tumor in nude mice. In an attempt to define the requirements of an intact T cell immunity for the observed protection against tumor, groups of C3H nu/nu were subjected to a pretreatment protocol with the four inactivating modalities identical to that used in the (AKR X B10.A)F1 mice. Mice were challenge six days after the last treatment. Table 5 shows that pretreatment with cells inactivated by X irradiation provided significant protection against subsequent challenge compared with untreated controls. Treatments with cells inactivated by 8-MOP/UVA, by mitomycin C, and by glutaraldehyde also seemed to protect a fraction of mice, but this was not significant considering the very small sample sizes. As was observed generally in (AKR X B10.A)F1 mice, initial development of a tumor mass at the site of challenge corresponded with eventual

death, usually within forty days. However, the development of a tumor mass in nude mice took a few days longer than in (AKR X B10.A) mice, with no tumor apparent by day 4 and little evident on day 7. For this reason, measurements were carried out until day 16, after which measurements were deemed to be unrepresentative of true systemic tumor burden due to ulcerations and necroses of the original cutaneous tumor masses. Unlike those in (AKR X B10.A) mice, tumors in nude mice never were observed to regress after initial appearance.

Retrospective analysis of this experiment revealed that it was poorly controlled due to the substantial difference in the ages of the experimental and control groups. Because of extremely limited availability of the C3H nu/nu mouse and backorders at its exclusive supplier, we were forced to accept the mice in stages, resulting in the use of three generations of nude mice in our experiment. At the time of challenge, five of the mice were approximately 27 weeks old; another five were approximately 14 weeks old; a further eight mice were approximately 11 weeks old; and the final five mice were approximately 10 weeks old. These final five mice, which arrived after the experimental mice had begun to receive treir treatments, were used as the control mice. Thus, the control mice and eight of the experimental mice were 16 to 17 weeks younger than the five oldest mice in the experiment. Although an attempt was made to randomize the old and young mice among the different experimental groups, the random assignments were necessarily uneven owing to the odd numbers of mice in each age group. As is discussed below, nude mice have been found to accumulate with age increasing numbers of

cytolytically active Thy-1 +, CD8+ cells (65), adding a significant variable to nude mouse experiments in which experimental and control groups are not age-matched. The lack of proper controls, however, does not explain the enhanced efficacy of X-irradiation-inactivated cells in immunizing nude mice compared with (AKR X B10.A)F1 mice, nor the delay in tumor formation at the site of inoculation of tumor cells. Therefore, these data suggest the operation of a mechanism of anti-tumor immunity in nude mice that is wholly different from, or, perhaps, an occult component of,the mechanism of immunity in immunologically normal mice. These experiments, however, must be repeated with fully age-matched nude mice before arriving at any firm conclusions.

2B4.11-immune mice are not significantly cross-protected against C10.9. Table 6 shows the results of in vivo specificity assays in which mice were pretreated with 8-MOP/UVA-inactivated 2B4.11 cells and subsequently challenged either with 2B4.11 or with C10.9, a T cell hybridoma differing from 2B4.11 by its TCR specificity, but still genetically compatible with the hybrid mice. Once again, 2B4.11-pretreated mice were significantly protected from tumor growth and mortality after challenge with 2B4.11. 2B4.11-pretreated mice challenged with C10.9, however, experienced a tumor growth and death rate not significantly different from those of untreated control mice. The minor degree of protection against C10.9 in 2B4.11-treated mice might achieve statistical significance with larger sample sizes. For this reason, identical experiments are currently underway, as well as experiments in which the pretreating and challenging cells include

not only C10.9, but also the parental thymoma BW5147 and 2B4.11.21.2.2, the TCR negative variant of 2B4.11.

Splenocytes from 2B411-immune mice demonstrate no in vitro cytotoxic activity. Despite good labelling, with maximal release ranging from 2000 to 7000 cpm, and spontaneous release consistently below twenty percent of maximum release, our cytotoxicity assays (data not shown) failed to show any cytotoxic activity among splenocytes taken from 2B4.11-immune mice. Attempts were made to demonstrate cytotoxic activity from effector cells directly after one passage of in vitro stimulation. In addition, animals were boosted with 8-MOP/UVA-inactivated 2B4.11 cells prior to harvest of splenocytes as a means of in vivo stimulation of effector cells. Nevertheless we were unable to bring forth evidence of cytotoxic activity in the splenocytes of 2B4.11-immune mice. These results lead us to conclude that in vivo sensitization may not be sufficient and that more than one passage in vitro may be necessary to bring forth any cytotoxic population. However, we cannot exclude the possibility that a cytotoxic cell might be present outside of the spleen within the lymph nodes. Nor does the failure to demonstrate cytotoxicity in these assays rule out the possibility of an antibody-dependent cell-mediated cytotoxic mechanism operating in our immune mice.

TABLE 1

Inactivation of 2B4.11 by 8-MOP/UVA
[^{3}H] Thymidine Incorporation (CPM x 10^3 ± SD)

[8-MOP] (ng/ml)	UVA (J/cm^2)	Day 0	Day 3	Day 6	Day 8	Day 10
none	none	155.5 ± 25.8	167.4 ± 23.8	193.4 ± 20.2	209.7 ± 7.3	253.0 ± 57.7
none	1	9.5 ± 0.2	102.8 ± 6.6	ND	ND	ND
none	3	6.2 ± 0.3	87.9 ± 9.3	ND	ND	ND
100	0	86.3 ± 15.0	112.6 ± 12.3	68.2 ± 23.4	81.7 ± 16.9	ND
100	2	16.0 ± 1.2	15.5 ± 1.0	88.8 ± 6.1	79.6 ± 12.5	ND
100	3	16.8 + 0.5	111.3 + 10.1	85.9 + 9.4	96.6 + 23.3	ND
100	7	2.9 ± 0.6	1.3 ± 0.9	1.3 ± 0.6	7.3 ± 1.9	3.8 ± 2.2
200	0	130.0 ± 12.9	131.0 ± 25.8	220.9 ± 40.1	248.4 ± 21.2	240.4 ± 14.6
200	2	14.5 ± 3.9	1.0 ± 0.1	1.9 ± 0.2	8.4 ± 3.7	4.6 ± 0.4
500	0	156.2 ± 15.2	179.2 ± 33.1	237.1 ± 27.0	243.4 ± 25.9	269.5 ± 20.1
1000	0	146.8 ± 8.6	198.3 ± 35.6	154.9 ± 47.4	235.0 ± 58.6	259.0 ± 39.1

ND = Not Done

TABLE 2

Inactivation of 2B4.11 by Mitomycin C, Glutaraldehyde, and X-irradiation
[^{3}H] Thymidine Incorporation (cpm ± SD)

Method of Inactivation	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10
Experiment 1						
None	130572 ± 26015	ND	185576 ± 14322	201806 ± 11696	160759 ± 56425	177647 ± 71684
Mitomycin C 50 ug/ml	61936 ± 8507	ND	3945 ± 1192	1936 ± 1052	1152 ± 597	1132 ± 673
Experiment 2						
None	41523 ± 2949	106709 ± 10308	ND	ND	ND	ND
Glutaraldehyde 0.5%	386 ± 119	377 ± 79	368 ± 61	189 ± 33	269 ± 37	ND

TABLE 3

Short Term Immunity[a] Against Tumor
by Pretreatment[b] with Variously Inactivated 2B4.11 Cells

Method of inactivation	Tumor Area[c], Days After Challenge (mm^2 ± SEM) (Number with tumors/Number challenged) Day 4	Day 7	Day 10	Dead at 40 days	Dead at 80 days
None (control) n=10	36 ± 6 (9/10)	123 ± 38 (10/10)	157 ± 23 (10/10)	9/10	10/10
8-MOP/UVA n=12	24 ± 3[d] (7/12)	53 ± 8 (8/12)	141 ± 29 (6/12)[e]	6/12[f]	6/12[f]
Mitomycin C n=10	31 ± 6 (8/10)	72 ± 17 (6/10)	129 ± 56 (5/10)[e]	4/10[f]	6/10[f]
Glutaraldehyde n=11	36 ± 4 (10/11)	76 ± 9 (11/11)	84 ± 29 (7/11)	6/11	8/11
X-irradiation n=12	28 ± 4 (10/12)	58 ± 9 (9/12)	145 ± 35 (8/12)	5/12[f]	9/12

a - Challenged six days after last treatment by s.c. injection of 5×10^6 viable 2B4.11 cells.

b - Pretreatment by four weekly i.p. injections of 5×10^6 inactivated 2B4.11 cells.

c - Mean of tumor areas on tumor-bearing mice only.

d - Significant suppression of tumor growth versus control ($p=0.04$)

e - Significant decreases in tumor incidence versus control ($p < 0.05$)

f - Significant increase in survival versus control ($p < 0.05$)

TABLE 4

Long Term Immunity[a] Against Tumor
by Pretreatment[b] with 2B4.11 Cells Inactivated by 8-MOP/UVA

Method of inactivation	Tumor Area[c], Days After Challenge (mm^2 ± SEM) (Number with tumors/Number challenged) Day 4	Day 7	Day 10	Dead at 40 days	Dead at 80 days
None (control, not pretreated) n=6	46 ± 9 (5/6)	164 ± 72 (5/6)	175 ± 10	4/6	6/6
8-MOP/UVA n=8	25 ± 5 (5/8)	63 ± 16 (3/8)	118 ± 8[d] (2/8)	0/8[e]	1/8[f]

a - Challenged one month after last treatment by s.c. injection of 5×10^6 viable 2B4.11 cells.

b - Pretreatment by four weekly i.p. injections of 5×10^6 inactivated 2B4.11 cells.

c - Mean of tumor areas on tumor-bearing mice only.

d - Significant suppression of tumor growth versus control ($p=0.02$)

e - Significant increase in survival versus control ($p < 0.01$)

f - Very significant increase in survival versus control ($p < 0.05$)

TABLE 5

Tumor immunity[a] in C3H nu/nu athymic mice
Pretreated[b] with Variously Inactivated 2B4.11 Cells

Method of inactivation	Tumor Area[c], Days After Challenge (mm^2 ± SEM) (Number with tumors/Number challenged) Day 4	Day 7	Day 10	Day 16	Dead at 40 days	Dead at 80 days
None (control, not pretreated n=5	nil (0/5)	23 ± 7 (2/5)	124 ± 26 (4/5)	322 ± 61 (5/5)	5/5	5/5
8-MOP/UVA n=4	nil (0/4)	nil (0/4)	42 (1/4)	227 ± 14 (2/4)	2/4	2/4
Mitomycin C n=5	nil (0/5)	nil (0/5)	115 ± 37 (3/5)	253 ± 65 (4/5)	3/5	4/5
Glutaraldehyde n=5	nil (0/5)	49 (1/5)	137 ± 59 (3/5)	208 ± 128 (3/5)	1/5[e]	4/5
X-irradiation n=4	nil (0/4)	nil (0/4)	nil (0/4)[d]	101 (1/4)[d]	0/4[e]	1/4[e]

a - Challenged six days after last treatment by s.c. injection of 5×10^6 viable 2B4.11 cells.

b - Pretreatment by four weekly i.p. injections of 5×10^6 inactivated 2B4.11 cells.

c - Mean of tumor areas on tumor-bearing mice only.

d - Significant decrease in tumor incidence versus control ($p=0.05$)

e - Significant increase in survival versus control ($p < 0.05$)

TABLE 6

SPECIFICITY OF TUMOR IMMUNITY IN MICE PRETREATED WITH 2B4.11 CELLS INACTIVATED BY 8-MOP/UVA AND CHALLENGED WITH VIABLE 2B4.11 OR C10.9 CELLS

PRETREATMENT[a]	CHALLENGE[b]	Tumor Area[c], Days After Challenge (mm^2 ± SEM) (No.with tumors/No. challenged) Day 4	Day 7	Day 10	Dead at 40 days	Dead at 80 days
None (control) n=5	2B4.11	33 ± 3 (5/5)	83 ± 6 (5/5)	141 ± 8 (5/5)	4/5	5/5
None (control) n=5	C10.9	22 ± 4 (5/5)	49 ± 8 (5/5)	66 ± 11 (5/5)	2/5	5/5
2B4.11 n=6	2B4.11	19 ± 5[d] (3/6)	51 ± 9[d] (3/6)	50+/-24 (4/6)	3/6	3/6
2B4.11 n=6	C10.9	19 ± 3 (5/6)	41 ± 2 (5/6)	66 ± 4 (5/6)	3/6	4/6

a - Pretreatment by four weekly i.p. injections of $5x10^6$ inactivated 2B4.11 cells.

b - Challenged six days after last treatment by s.c. injection of $5x10^6$ viable 2B4.11 cells or of $10x10^6$ viable C10.9 cells.

c - Mean of tumor areas on tumor-bearing mice only.

d - Significant suppression of tumor growth versus control (p=0.04).

DISCUSSION

Immunity against tumor in murine experimental systems has been the object of study for over fifty years. Initial investigations surrounded the observation that chemically-induced sarcomas often were rejected when transplanted into syngeneic animals (67). Later it was observed that immune reactions against highly malignant B cell tumors could be demonstrated in mice in which established tumors had been excised surgically or treated by chemotherapy (68, 69). Other investigators found that immunity against a plasmacytoma could be induced in mice by prior inoculation with irradiated plasmacytoma cells (70). This immunity, which could be transferred via splenocytes to syngeneic animals, was thought to reside principally in the CD8+ cytotoxic T cell population. Other studies, however, demonstrated the contribution of humoral immunity (71), natural killer (NK) cell activity (72, 73)), and lymphokine-activated killer (LAK) activity (74) to the phenomenon of anti-tumor immunity.

Our experiments demonstrate that it is possible to induce immunity against a T cell tumor by pretreating mice with inactivated tumor cells. These results add to the model of immunity against T cell tumor established by Ashwell et al. (18) and by Bridges and Longo (64) by showing that T cell hybridomas can be immunogenic when inactivated by means other than occupancy of the T cell receptor by antigen. Although our results indicate that all tested forms of inactivation produced anti-tumor "vaccines" of varying degrees of efficacy, it appears that cells inactivated by

8-MOP/UVA conferred the most protection against tumor development and tumor-related mortality. The apparently superior immunogenicity of 8-MOP/UVA-inactivated cells may be related to a unique mechanism of inactivation, or to the induction of a unique set of targets and effectors of the immune response.

The finding that cells inactivated by the bifunctional alkylating agent mitomycin C were nearly as effective as cells inactivated by 8-MOP/UVA in immunizing mice against tumor suggests that the well characterized DNA crosslinking abilities of UVA-activated 8-MOP in some way also enhances the immunogenicity of inactivated cells. While the purely cytotoxic effects of 8-MOP/UVA can be attributed to the crosslinking of nuclear DNA, a putative alteration in the immunogenicity of cells after 8-MOP/UVA treatment raises the possibility that cell-membrane DNA (cmDNA) is a crucial substrate for 8-MOP/UVA adduct formation. Crosslinking of cmDNA theoretically could alter cell-surface stereochemistry, perhaps making available previously occult or wholly novel immunogenic molecules to the autologous immune system. Support for the role of cmDNA in the mechanism of photopheresis has come from recent findings by Perez et al. (75). Using skin allograft rejection between histoincompatible mice to provoke a T cell lymphoproliferative response, her group found that effector lymphocytes incubated in DNase were as effective as those inactivated by 8-MOP/UVA in inducing suppression of effector lymphocyte function in delayed type hypersensitivity assays, suggesting that the removal of cell surface DNA could reproduce certain aspects of the actions of UVA-activated 8-MOP.

Cell-surface modifications are almost certainly not limited to cmDNA. As discussed above, UVA-activated 8-MOP is capable of interacting with protein and lipid molecules. UVA-activated 8-MOP forms extensive covalent bonds with cellular proteins (76) and is known to oxidize and to form cycloadditions with lipids (77). The potential for modulation in the antigenicity of cells by reactions with cell membrane lipids and proteins, such as histocompatibility antigens, is enormous. It is clear that the wide distribution and interactions of UVA-activated 8-MOP will make it difficult to reduce the immunological phenomena associated with their use to precise molecular processes. Given the diverse sites of reactions by 8-MOP, it is not unreasonable to hypothesize that 8-MOP might be inactivating 2B4.11 by mimicing T cell receptor-mediated activation, perhaps by a currently uncharacterized interaction with the second messenger systems that mediate T lymphocyte activation. Investigation of this possibility will require assays for interleukin 2 (IL-2) production and for evidence of phosphorylation events signalling the activation of second messenger systems after exposure to UVA-activated 8-MOP.

Another intriguing possibility for the target of 8-MOP comes from the mutagenesis experiments of Boon et al. with the mouse mastocytoma P815 (78). When subjected to mutagenic chemicals, this nonimmunogenic and highly tumorigenic cell line has formed variants that lose the tumorigenicity of their parent cells, becoming so-called "tum-," while simultaneously gaining expression of tumor rejection antigens. These tum- variants can induce an immune protection against the original parent tumor cells mediated by

class I-restricted cytolytic T lymphocytes (79). Each of these tumor rejection antigens was found to be a unique molecule arising from a single point mutation in a normal allele (80,81,82) or from increased expression of a normal but largely silent gene (83). Preliminary findings by Gasparro et al. indicate that UVA-activated 8-MOP is itself highly mutagenic (unpublished data), lending credibility to the notion that "tum-" variants might be created by the process of photopheresis. It would seem improbable, however, that such variants were generated during the 8-MOP/UVA protocol used in our experiments, as the titration curves show that with the dose employed for 2B4.11 inactivation, there was no evidence of cell recovery for up to ten days after exposure to 8-MOP/UVA. In addition, the lack of tumor development and death during the treatment protocol, among both immune competent and immune incompetent mice, provides strong evidence that there was no emergence of mutant cell populations.

The components of the immune system that mediate the immunity observed in our experiments cannot be identified directly from the present data. However, certain characteristics can be supposed or inferred both from our experiments and from the work of other investigators in this model. The observation that immunity against tumor was better at one month than at six days after the treatment protocol implies that the mechanism resides in a cell population or populations with immunological memory. This would include both T cell and B cell immunity.

On the other hand, our finding that nude mice treated with X irradiated tumor cells were protected against tumor formation

suggests at first glance that T cell mediated immunity may be of minor significance in our system. But, as previously mentioned, the substantial differences in age between mice in the experimental and control groups makes it difficult to reach valid conclusions using this experiment. It was reported recently that increasing numbers of Thy-1+ lymphocytes accumulate with age in the spleens and lymph nodes of nude mice, and that among these are found an unusually large number of constituitively cytolytic, CD8+, alpha/beta and gamma/delta TCR-bearing lymphocytes that resemble the intestinal epithelial lymphocytes of euthymic mice (66). The immunity induced in nude mice by our protocol may represent a reaction mediated by this set of T cells, by which the oldest mice in our experimental groups were able to mount an immune response against tumor. Because of these complexities of the nude mouse immune system, we now are seeking formal proof of simple involvement of the immune system by subjecting whole-body irradiated (AKR X B10.A)F1 mice to our treatment protocol, as others have demonstrated that mice subjected to a dose of 6Gy of gamma irradiation were more effectively immunosuppressed than nude mice (84).

Nevertheless, it is clear from the differing efficacies of X irradiated tumor cells as immunizing agents that the immune mechanism in our surviving nude mice is different from that in our surviving (AKR X B10.A)F1 mice. The immunity observed in nude mice may be mediated by their unique T cell population as previously described. But nude mice also have been reported to possess equivalent or greater numbers of B cells and NK cells than euthymic

mice of the same strain (84). It therefore is possible that the anti-tumor immunity in our nude mice represents a process characterized predominantly by NK cell activity, with or without antibody mediation. The apparent superiority of X irradiation as a form of tumor inactivation for immunizing nude mice may merely be an artefact of unequal distribution of the oldest mice among the experimental groups, with no inactivating modality uniquely enhancing the immunogenicity of the tumor cells. Conversely,our results may reflect a superior ability of X irradiation to render cells immunogenic to the peculiar immune system of the nude mouse.

While we have been as yet unable to isolate a cytotoxic cell population from our 2B4.11-immune mice, Bridges and Longo have established clearly that 2B4.11-specific cytotoxic CD8+ T lymphocytes are present in the spleens of 2B4.11-immune mice and that the CD8+ population is essential to the anti-tumor immunity induced by T cell receptor-mediated activation (64). These CD8+ lymphocytes were cytotoxic only for hybridoma 2B4.11 and were the only relevantly active cell type identifiable by in vitro studies.

Our studies of in vivo specificity of anti-tumor immunity demonstrated no significant cross-protection of 2B4.11-immune mice against challenge with C10.9. However, in the system of Bridges and Longo, mice rendered immune to 2B4.11 by T cell receptor occupancy also were protected against related hybridoma C10.9 and parental thymoma BW5147. Thus it was evident that despite in vitro specificity for 2B4.11, a less specific effector cell population was contributing to immunity in vivo, perhaps under the recruitment of DTH mechanisms used by CD4+ cells or by a recently described

tumor-specific DTH mechanism employed by a CD8+ population (85). Current experiments in our laboratory using C10.9, BW5147, and 2B4.11.21.2.2, the TCR-negative variant of 2B4.11, will determine whether the immunity provoked by 8-MOP/UVA-inactivated tumor cells is in some degree nonspecific *in vivo*.

It is also possible that the differences between *in vitro* and *in vivo* immunity represent the contribution of humoral immunity. Cihak *et al.* demonstrated the significant, but not essential, role of anti-tumor antibodies as an adjunct to cell-mediated immunity against a murine plasmacytoma (70). Such antibodies could mediate antibody-dependent cell-mediated cytotoxicity, providing for cell killing that is not restricted to MHC class I and hence would not be evident in *in vitro* assays of CD8+ lymphocyte-mediated cytotoxicity. We currently are studying serum from 2B4.11-immune mice for evidence of antibody-binding to 2B4.11 and for the ability to induce lysis of target cells when added to splenocytes taken directly from immune mice in cytotoxicity assays.

Final consideration must be given to the cells that have been found to mediate the suppression of T lymphoproliferative responses in previous animal models of photopheresis. As described above, Perez *et al.* demonstrated that the suppression of T cells mediating skin allograft rejection could be transferred to naive syngeneic animals via a Thy-1+, Lyt-2+, L3T4- lymphocyte population (60). Thus, 8-MOP/UVA-inactivated T lymphocytes can provoke a CD8+ suppressor cell response. It is possible that a suppressor, rather than a cytotoxic, cell is provoked by 8-MOP/UVA-inactivated 2B4.11 cells.

The lymphocyte subset depletion experiments used both by Bridges & Longo (64) and by Perez et al. (60) to determine the phenotype of the cells mediating the respective anti-T cell responses offer the best strategy for determining the identity of the cells mediating immunity against tumor in the current system. This can be performed either by an adoptive transfer, Winn assay system with depletion of lymphocytes during transfer of cells from immune to naive mice, or by in vivo depletion of lymphocytes prior to subjecting mice to the immunization protocol.

Knowledge of the cell type mediating the anti-tumor immunity will bring us a long way toward discovering the target of the immune response. For instance, proof of CD8+ lymphocyte mediation would identify the antigen as an MHC class I-associated peptide, perhaps the product of novel, 8-MOP/UVA-induced antigen processing, or an immunogenic alteration by 8-MOP/UVA of the resident peptide of the class I molecule. UVA-activated 8-MOP could mediate the cross-linking of class I molecules to create receptive peptide binding sites that recently have been reported to enhance immunogenicity (86). Predominantly humoral immunity would implicate an unprocessed molecule as the immunogen, perhaps an altered cell-membrane lipid, protein, or DNA molecule.

The 2B4.11-specific CD8+ cytotoxic lymphocyte response demonstrated by Bridges and Longo (64) raises the possibility that the T cell receptor itself can be processed for presentation to MHC class I-restricted lymphocytes. This correlates with the finding by Lider et al. (87) that vaccination against EAE-mediating T cell clones induced an anti-idiotypic response apparently directed

against the TCR of the immunizing clone. However, the anti-idiotypic response in the EAE model was mediated by a CD8+ suppressor cell. In addition, Sun et al. (88) described immunity against EAE-inducing clones that was mediated by CD8+ suppressor and cytotoxic lymphocyte lines. These CD8+ lines seemed to be capable of recognizing EAE-inducing clones in an anti-idiotypic manner, but without apparent MHC restriction, leading to speculation of a direct interaction between the TCR of the EAE-inducing clone and that of the protecting clone.

A further exciting possibility raised by studies in the EAE model is that of immunity against T cell activation antigens, or "anti-ergotypic" immunity, described by Lohse et al. (89). These studies showed that vaccination against EAE-mediating clones induced not only an anti-idiotypic T cell response, but also an anti-ergotypic T cell response to undefined, activation-associated changes in the EAE-mediating clones. These anti-ergotypic cells were found to be a phenotypic mixture of CD4+ and CD8+ T lymphocytes. The demonstration of specific immune reactions against T cell activation-associated antigens may provide a clue to the target of the immune reaction against T cell hybridomas upon their growth inhibition by T cell receptor-mediated activation. If T cell activation is among the effects of UVA-activated 8-MOP, then anti-ergotypic immunity also would provide a possible explanation for the immunogenicity of 8-MOP/UVA-treated tumor cells.

The results of these experiments demonstrate that 8-MOP/UVA is a method of cellular inactivation that is uniquely efficacious in inducing an immune response against the 2B4.11 T cell hybridoma.

It therefore is reasonable to view this system as a model that can be applied to study the immune mechanisms of photopheresis. In addition to the future experiments described above, we will attempt to make this model more akin to clinical photopheresis by studying the ability of 8-MOP/UVA-inactivated tumor cells to induce the regression of established tumors. We also will investigate the possible adjuvant effect of photoinactivated bystander lymphocytes,as they exist in the clinical setting of photopheresis. This will be accomplished by adding 8-MOP/UVA-inactivated syngeneic splenocytes to our immunization protocol, which currently uses pure, clonal populations of tumor cells. The specific molecular effects of UVA-activated 8-MOP also will be explored by determining the pattern of DNA adduct formation in 2B4.11, by probing for evidence of second messenger system activation via phosphorylation events, and by analyzing 8-MOP/UVA-treated cells for changes in expression of cell surface molecules such as MHC class I. By these means it may be possible eventually to complete the reduction of photopheresis from clinical phenomenology to molecular immunology.

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